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Energetic problems of extremely alkaliphilic aerobes

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Abstract

Over a decade of work on extremely alkaliphilic *Bacillus* species has clarified the extraordinary capacity that these bacteria have for regulating their cytoplasmic pH during growth at pH values well over 10. However, a variety of interesting energetic problems related to their Na^+-dependent pH homeostatic mechanism are yet to be solved. They include: (1) the clarification of how cell surface layers play a role in a category of alkaliphiles for which this is the case; (2) identification of the putative, electrogenic Na^+/H^+ antiporter(s) that, in at least some alkaliphiles, may completely account for a cytoplasmic pH that is over 2 pH units lower than the external pH; (3) the determination of whether specific modules or accessory proteins are essential for the efficacy of such antiporters; (4) the mechanistic basis for the increase in the transmembrane electrical potential at the high external pH values at which the potential-consuming antiporter(s) must be most active; and (5) an explanation for the Na^+-specificity of pH homeostasis in the extremely alkaliphilic bacilli as opposed to the almost equivalent efficacy of K^+ for pH homeostasis in at least some non-alkaliphilic aerobes. The current status of such studies and future strategies will be outlined for this central area of alkaliphile energetics. Also considered, will be strategies to elucidate the basis for robust H^+-coupled oxidative phosphorylation by alkaliphiles at pH values over 10. The maintenance of a cytoplasmic pH over 2 units below the high external pH results in a low bulk electrochemical proton gradient (Δp). To bypass this low Δp, Na^+-coupling is used for solute uptake even by alkaliphiles that are mesophiles from environments that are not especially Na^+-rich. This indicates that these bacteria indeed experience a low Δp, to which such coupling is an adaptation. Possible reasons and mechanisms for using a H^+-coupled rather than a Na^+-coupled ATP synthase under such circumstances will be discussed.

Keywords: Alkaliphile; ATP synthase; Sodium ion/proton antiporter; pH homeostasis; Cytochrome *c*; (*Bacillus*)

1. Introduction

Extremely alkaliphilic aerobic bacteria are primarily *Bacillus* species that are neither thermophiles nor particularly dependent upon substantial concentrations of added Na^+ for growth [1]. In those instances where rigorous attempts have been made to minimize contaminating Na^+, a dependence of growth on Na^+ has been shown [2]. It is not clear, however, that this requirement extends to all alkaliphilic *Bacillus* species, or always obtains when glucose rather than non-fermentative carbon sources are used to support growth. Quite likely, Na^+ will be required for co-transport of vital minerals or trace amino acids and vitamins since the ion/solute symport systems of alkaliphilic *Bacillus* species are consistently found to be Na^+-coupled [3]. For at least some strains, and perhaps all, Na^+ is also specifically required for active pH homeostasis at elevated pH [4–7].

There is a category of alkaliphiles in which the capacity for alkaliphily apparently depends upon specific properties of cell wall layers [8]. Cell walls rich in uronic acid and/or aspartic and glutamic acids are important for alkaliphily in some groups of *Bacillus* species. A highly negatively charged cell wall may be involved in exclusion of hydroxyl ions. Active Na^+-dependent pH homeostatic mechanisms involving antiporters are apparently important to this group of alkaliphiles in addition to the role of the cell wall components (see discussion of *Bacillus* C125 below). Perhaps an optimal adaptation to high external pH values will include some as yet undetected cell surface properties even in extreme alkaliphiles, such as *Bacillus firmus* OF4, whose cell wall layers do not seem very

Abbreviations: Δp, transmembrane electrochemical proton gradient; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential.

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different from those of *Bacillus subtilis* [8,9]. Will, however, the pH homeostatic mechanisms work against a smaller burden in alkaliphiles that are particularly reliant upon certain cell wall layer structures? In other words, can such structures provide a meaningful, even if partial, barrier against the external pH or allow a higher concentration of protons near or within the cytoplasmic membrane? If so, an antiporter that serves the needs of pH homeostasis in *Bacillus* C125 might not serve those needs completely in *B. firmus* OF4. Perhaps recently used pH probes could also be adapted to approach such questions [10]. It may further be instructive to compare the secreted proteins or external loops of membrane proteins from different categories of extreme alkaliphiles. In general, these proteins or protein segments have a paucity of basic amino acids relative to homologues from non-alkaliphilic bacilli [11–14]. If the cell wall layers of some alkaliphile strains allow the membrane-proximal area to be significantly more acidic

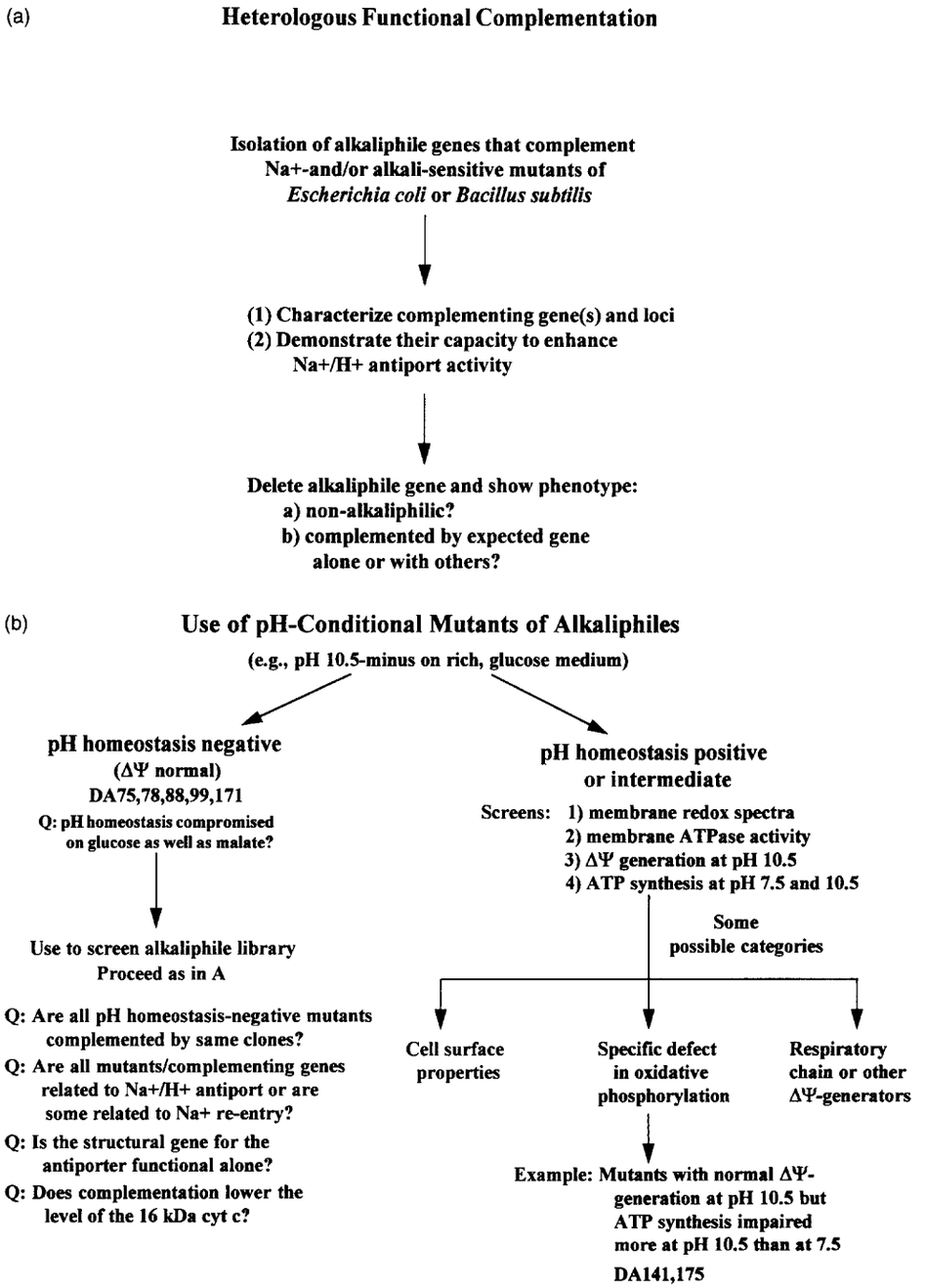

Fig. 1. A schematic approach to identify Na^+/H^+ antiporter or related genes from alkaliphilic aerobes (A) and for the analysis of non-alkaliphilic mutants of alkaliphilic aerobes with defects in pH homeostasis or in oxidative phosphorylation or related problems such as in $\Delta\psi$ generation (B).

Table 1
pH Homeostasis in *B. firmus* OF4 and *B. subtilis* BD99 in the presence of Na^+ or K^+

Strain	Cytoplasmic pH after pH shift			
	pH 7.5 → 8.5 [a]		pH 8.5 → 10.5 [b]	
	K^+	Na^+	K^+	Na^+
B. firmus OF4	8.5	7.3	10.5	8.3
B. subtilis BD99	7.4	7.3	10.5	10.5

[a] Cells were equilibrated at pH 7.5 in either 100 mM potassium or sodium phosphate. The external pH was abruptly raised to pH 8.5 by dilution into either potassium or sodium phosphate. The cytoplasmic pH was determined after 10 min from the distribution of ^{14}[C]methylamine.
[b] Cells were equilibrated at pH 8.5 in either 100 mM potassium or sodium phosphate. The external pH was raised to 10.5 by dilution into either potassium or sodium carbonate. The internal pH was determined after 10 min by the distribution of ^{14}[C]methylamine.

than the bulk external pH, then this adaptation might be absent from the membrane proteins of such strains but not from the proteins secreted into the bulk.

2. Na^+-dependent pH homeostasis in *Bacillus firmus* OF4 and *Bacillus* C125

This laboratory has worked principally on alkaliphilic *B. firmus* OF4, a facultative alkaliphile that grows on malate from pH 7.5 to at least pH 11.2 in continuous culture with rigorously maintained external pH; growth at pH 10.5 occurs at a faster doubling rate than at pH 7.5 [15]. The cell wall layers of *B. firmus* OF4 are, to the first approximation, comparable to those in *B. subtilis* [9]. In *B. firmus* OF4 the ability to maintain a cytoplasmic pH below 9.0 is associated with optimal growth. In malate-containing media, this capacity exists up to pH 11.2 [15], at which point the pH gradient, ΔpH acid in, is 2.3 units. At the higher external pH value of 11.4, the magnitude of the pH gradient falls below two units and correlates with a dramatic decrease in growth rate. The upper pH limit of alkaliphile growth on malate, above pH 11, thus appears to reflect the maximal ΔpH acid in, that can be sustained by the pH homeostatic mechanism — i.e., about 2.3 units. Abundant evidence supports the absolute dependence of this mechanism on Na^+ [3]. As shown in Table 1, the specific requirement by *B. firmus* OF4 for Na^+ for pH homeostasis is observed even upon a modest shift in external pH from 7.5 to 8.5. This is in contrast to *B. subtilis*, which can use either Na^+ or K^+ for pH homeostasis, although neither one is effective much above pH 8.5 (Table 1 and Ref. [5]). A study of a different facultative alkaliphile, *Bacillus* YN-2000, had shown that added K^+ actually compromised Na^+-dependent pH homeostasis [16], and another study of an obligate alkaliphile indicated that at least in the absence of added Na^+, K^+ alkalinized the internal pH [17]. The ion specificity of alkaliphiles for pH homeostasis could indicate that alkaliphiles may be more at risk of losing internal K^+ than neutralophiles and/or that maintenance of high K^+/Na^+ ratios is more critical for proper functioning of cytoplasmic enzymes in alkaliphiles. Na^+ toxicity in some systems is known to increase as the pH rises and Na^+ toxicity is correlated, at least in part, to a decreased ratio of cytoplasmic K^+/Na^+ [18–20].

Abundant evidence also supports the hypothesis that the mechanism of Na^+-dependent pH homeostasis is the use of antiporter(s) that catalyze electrogenic exchange of cytoplasmic Na^+ for extracytoplasmic H^+, energized by the Δp established by a proton-extruding respiratory chain [12]. We have most recently isolated a large panel of non-alkaliphilic mutant strains of *B. firmus* OF4 (DA strains) (Fig. 1) that were isolated on the basis of their inability to grow on rich media at pH 10.5 after mutagenesis of wild-type cells with 1-methyl-3-nitro-1-nitrosoguanidine. Five such strains (DA75, 78, 88, 99, 171) were totally unable to regulate their cytoplasmic pH upon an 8.5 to 10.5 shift in external pH, although their capacity to generate the respiration-dependent driving force for antiport activity was normal. All five strains exhibited increased sensitivity to high Na^+ concentrations. Interestingly, all but one of those five DA strains, but not other categories of non-alkaliphilic strains (see below), had elevated levels of *c*-type cytochromes relative to *a*- and *b*-type cytochromes. The content of heme-stained polypeptides separated by SDS PAGE was examined in one mutant, DA99; one of the four cytochrome *c* species identified in the wild type strain [12] was clearly elevated. Perhaps that cytochrome *c* species, a species with an apparent molecular weight of 16 kDa for which no function had yet been assigned [12], is part of a pH sensor device or network in the alkaliphile. Membrane vesicle assays will be used to demonstrate the correlation between the loss of pH homeostatic capacity and a diminished activity of Na^+/H^+ antiport [21,22]. These assays may reveal some difference between the four strains with an elevated cytochrome *c* ratio and the one strain that did not show such an increase.

The DA78 strain will be used to screen libraries of *B. firmus* OF4 for complementing genes, as indicated in Fig. 1B. Candidates for antiporter-encoding genes with a major role in pH homeostasis would be identified by sequence analysis and their restoration of antiport activity to the DA strain. A single antiporter-encoding gene, the *tetA*(L) gene of *B. subtilis*, has recently been shown to have a dominant role in pH homeostasis (both Na^+- and K^+-dependent) [23]. Perhaps there is a comparably dominant gene in *B. firmus* OF4, accounting for the ease and frequency with which non-alkaliphilic mutants were found that lacked the capacity for pH homeostasis. However, if more than one antiporter were required, then the complementation by any one gene will not completely restore the wild type phenotype. As an adjunct to such complementation tests of the DA strains it may thus be of value to screen putative

antiporter genes for their ability to restore pH homeostasis to a *tetA*(L) deletion strain, JC112, of *B. subtilis* [24] (Fig. 1A).

Skulachev [25] has suggested that use of Na^+/H^+ antiporters by an alkaliphile for pH homeostasis at very high pH would be problematic because of the low bulk Δp that would obtain as the pH rose. Such an energetic problem is compounded by the observation that pH homeostasis can be achieved by *B. firmus* OF4, for example, when a substantial, inwardly directed Na^+ gradient exists at high pH [3]. It is important to consider the possibility that the antiporter(s) that generate the two unit pH gradient in the alkaliphile has some special adaptation that ameliorates the thermodynamic challenge of the inwardly directed Na^+ gradient, the outwardly directed H^+ gradient, and a finite, although substantial $\Delta\psi$. Accessory proteins or specific antiporter domains might, for example, serve as devices for raising cationic substrate concentrations near the antiporter.

3. Status of the molecular characterization of the alkaliphile antiporters

The *nhaC* gene of *B. firmus* OF4 was isolated by functional complementation of the Na^+-sensitive phenotype of Na^+/H^+ antiporter-deficient strains of *Escherichia coli* [26]; the Na^+/H^+ antiport activity in the membranes of the complemented strains was increased upon *nhaC* expression. The *E. coli* strains are not, however, alkali-sensitive in the absence of added Na^+ [27]. Perhaps this is because they can use K^+ for pH homeostasis mediated by distinct K^+/H^+ antiporters like some apparent second-site mutants of *B. subtilis* JC112 [24]. A recombinant vector expressing *nhaC* was recently found not to complement DA78 or JC112 at elevated pH. Thus the NhaC-mediated antiport is competent for enhancing Na^+-resistance at near neutral pH, but not competent for substantial restoration of a capacity for pH homeostasis at pH values above 8. We are currently investigating whether this latter failure results from some feature of the fluxes catalyzed or a requirement for accessory proteins.

In an approach comparable to that to be used with the *B. firmus* OF4 DA strains, Kudo et al. [28] isolated non-alkaliphilic mutant strains of *Bacillus* C125. This strain is a facultative alkaliphile that normally grows in a pH range from 7 to 11, and is in the category of alkaliphiles that have high concentrations of cell wall uronic acid and glutamic acid [29]. Two different clones were found to complement two different non-alkaliphilic mutant strains [28,30]. One of the mutants thus complemented was shown to be deficient in Na^+/H^+ antiport activity [31]. The defect was complemented by a clone that encoded part or all of a putative antiporter gene. The C125 gene thus becomes the first alkaliphile gene to complement a non-alkaliphilic mutant of an alkaliphile. This C125 gene has significant homology with a recently completed and extended sequence for the *B. firmus* gene *nhaC* [32]. At the N-terminus of the deduced C125 gene product, however, there is a domain that is not found in *nhaC* but that shows significant sequence similarity to a hydrophobic region of chain-5, a subunit of the large, H^+-coupled NADH dehydrogenase complex [31].

The complementation of the C125 mutant occurred by a crossover such that all or part of the complementing gene repaired and rescued the mutated chromosomal gene [31]. It remains possible that the C125 gene is necessary but not sufficient for pH homeostasis and that polar effects of the mutation on several downstream genes [31] are relevant to the phenotype and could be corrected by the complementing crossover. To test for polar effects, it would be desirable to generate a disruption or deletion in a gene of interest. This would clarify whether the putative structural gene for the antiporter can functionaly complement alone or requires one or more accessory proteins. In *B. firmus* OF4, the protocols for making such targeted disruptions have now been developed and applied to *nhaC* disruption. The resulting strain has a Na^+- but not alkali-sensitive phenotype [32], consistent with the failure of the cloned *nhaC* gene to complement the JC112 strain of *B. subtilis* for alkali-sensitivity.

4. The problem of $\Delta\psi$ generation at pH > 9

If some sort of specialized devices are used to minimize the energetic burden against which the Na^+/H^+ antiporters of the extreme alkaliphiles function, there still is expected to be enhanced cost associated with electrogenic, $\Delta\psi$-consuming antiport activity at pH values above 8.5. This will be compounded in cells carrying out oxidative phosphorylation. Regardless of how the alkaliphile solves the problem of the low Δp vis à vis H^+-coupled ATP synthesis, the participation of a proton in the reaction chemistry ensures that there will be an added energetic cost at elevated pH [33]. Nonetheless, the $\Delta\psi$ of malate-grown cells rises over the pH range from 7.5 to 11.2 in pH-controlled chemostat cultures [15], and similar findings have been made with other alkaliphiles in this and other laboratories [7,34,35].

Several respiratory chain components are present in higher concentration in cells grown at high pH relative to near neutral pH [36,37] and the elevation of these components may be related to the higher $\Delta\psi$. It is further possible that some of these complexes are activated or better coupled to proton-translocation as the pH rises up to pH 10.8. Additionally, it is possible that not all of the pH-dependent increase in the $\Delta\psi$ is accounted for by respiration-driven proton translocation. Although there are respiration-coupled Na^+ extrusion systems in some marine bacteria [38], no compelling evidence for such complexes has emerged in numerous studies of respiration in non-marine extremely

alkaliphilic bacilli [3,39]. Nor has any Na^+-dependent component been observed for the membrane-associated ATPase activity of a few such organisms [40,41]. However, it remains possible that alkaliphiles might possess a low level of a primary system, such as the ABC-type Na^+-translocating transporter recently identified in *B. subtilis* and found to contribute to Na^+-resistance and secondary (presumably $\Delta\psi$-driven) accumulation of K^+ when the Δp is low [42]. It must be emphasized that if a primary Na^+ extrusion system does in fact function in extreme alkaliphiles, it would further increase the inwardly directed Na^+ gradient that is adverse for antiport function. Inasmuch as pH homeostasis seems to be the central problem for alkaliphiles, such extrusion systems would have limited utility — e.g., perhaps enhancing Na^+/solute symport at intermediate pH values or conditions in which the solute concentration rather than pH was growth limiting. Similarly, an ATP-dependent system for Na^+ extrusion would not alleviate the energetic dilemma connected to ATP synthesis via a H^+-translocating ATP synthase since ATP would be consumed to generate the increased $\Delta\psi$. Some DA strains exhibited lower capacity than wild type for $\Delta\psi$ generation; equivalent mutant strains, generated by transposon mutagenesis, may provide a way of identifying any adjunct primary pumps that contribute significantly to growth of the alkaliphilic bacilli under specified conditions.

5. The problem of oxidative phosphorylation at pH > 9

This problem will be briefly summarized in connection with the new DA mutants since a recent review has been presented [43]. In both continuous cultures and batch cultures of alkaliphilic *B. firmus* OF4 and in batch cultures of *Bacillus alcalophilus*, the highest phosphorylation potentials or [ATP]/[ADP] ratios are observed at high pH values at which the Δp is submaximal [9,15,44]. Although we had anticipated that Na^+ would be the coupling ion for the ATP synthase, just as it is for solute uptake, this is not the case. As in *Bacillus stearothermophilus*, which uses Na^+-coupled solute symport at high temperature [45], a solution for oxidative phosphorylation has been found without by-passing a H^+-coupled ATP synthase. The ATP synthases of both *B. firmus* OF4 [40] and *B. alcalophilus* [41] have been purified and shown to reconstitute H^+- but not Na^+-translocation, and *in vivo* studies in *B. firmus* OF4 are consistent with an exclusively H^+-coupled mode of ATP synthesis [46]. The reason for maintaining use of a H^+- rather than Na^+-coupled ATPase by both thermophilic and alkaliphilic aerobes when the Δp is sub-maximal is not yet clear. Since a heterologous Na^+-coupled ATPase can support growth of *E. coli* on succinate [47], such enzymes must be kinetically competent for oxidative phosphorylation at maximal Δp levels. The possibility that such enzymes might still be kinetically inadequate at low Δp relative to the H^+-coupled forms is worth investigation, since it might provide insights into the solution actually adopted by the alkaliphiles. One suggestion has been that at the high end of the pH range, the alkaliphile might couple protons to ATP synthesis with a higher H^+/ATP ratio than at lower pH [48], but this would require a variable stoichiometry, and also seems counter to the finding of the higher molar growth yields of *B. firmus* OF4 at the high pH values at which ATP synthesis was best but the Δp was low [15]. Additional observations indicate that artificially imposed diffusion potentials cannot energize ATP synthesis above pH 9 even though they are the same magnitude as respiration-generated potentials and can drive other $\Delta\psi$-dependent processes [9]. A number of models have been proposed in which the protons derived from one or more respiratory chain complexes might be sequestered at pH > 9 so that synthesis occurs without equilibration of a subset of such protons with the bulk [43]. Such a mechanism might depend upon: (i) protein–protein interactions at high pH allowing a direct proton transfer from pumps to synthase; (ii) structural features of the pumps or ATPase that could foster such interactions, e.g., specific sequence motifs that have been identified in F_0 subunits of several aerobic alkaliphilic *Bacillus* species; (iii) membrane surface properties promoting protein–protein interactions or obviating the need for them by sequestering protons on the lipid surface; or (iv) specific protein coupling factors for proton translocation to the synthase at high pH.

If there are specific membrane lipids or coupling factors involved in ATP synthesis at pH > 9 but not at lower pH values, then genes involved in this process might be identified by knock-out mutations such as transposon mutagenesis (procedures for generating knock-out mutations have recently been established in *B. firmus* OF4 [32]). However, if sequence motifs of the F_0 promote protein–protein interactions between the ATP synthase and a proton-translocating respiratory chain complex at pH > 9, knock-out mutations would not identify the particular sequence motifs involved. There might be, however, non-alkaliphilic mutants generated by chemical mutagenesis or site-directed mutagenesis that lost the capacity for ATP synthesis at pH > 9 while retaining normal pH homeostasis, $\Delta\psi$ generation, and relatively greater capacity for ATP synthesis at pH 7.5. A few such strains have been identified among the DA strains (DA141 and 175) along with others that have reduced synthesis and $\Delta\psi$ in spite of normal cytochrome profiles; these latter strains (DA157 and 164) may carry mutations that make the ATP synthase leaky to protons, similar to the DL-54 mutation of the *E. coli* synthase [49]. Mutagenized cells may, however, be found to possess multiple mutations or to have mutations in extracytoplasmic loops of important membrane proteins that adversely introduce a basic residue. Thus the analysis of pertinent DA strains will be accompanied by selected attempts to generate site-directed mutants with alterations

in alkaliphile-specific F_0 motifs. It will be important to correlate genetic work with biochemical assessments of possible protein–protein interactions that could permit a proton translocation between membrane complexes or other types of sequestration that might clarify the alkaliphiles' approach to oxidative phosphorylation.

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